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Sickling of sickle erythrocytes does not alter phospholipid asymmetry

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Experiments in which phospholipase A_2 has been used to examine the accessibility of phospholipids on the surface of sickled erythrocytes and of spectrin-free spicules derived from these cells have shown that accessibility is essentially unchanged compared with oxygenated sickle or normal erythrocytes. These results conflict with the claims of other workers that sickling is accompanied by loss of lipid asymmetry and that spectrin is important in maintaining the normal distribution of phospholipids in the erythrocyte membrane.

The erythrocyte plasma membrane displays a characteristic asymmetry in the transbilayer distribution of its phospholipids, with aminophospholipids mainly confined to the inner (cytoplasmic) leaflet of the bilayer and choline phospholipids accounting for most of the outer-leaflet lipid (Zwaal *et al.*, 1975; Op den Kamp, 1979; van Deenen, 1981). This distribution seems to be preserved throughout the life of the cell (~100 days), and it has been recently suggested that phospholipid asymmetry may be stabilized by specific interactions between aminophospholipids and membrane skeletal proteins, especially spectrin (Haest *et al.*, 1978; Haest, 1982; Kumar & Gupta, 1983). If alterations in the association of cytoskeletal elements with the plasma membrane could change the transbilayer distribution of phospholipids with the resulting appearance of phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the outer surface of the cell, this could have important biological and clinical consequences. In particular, it has been suggested that changes in the interaction of skeletal proteins with phospholipids may cause alterations in surface phospholipids that result in the enhanced clotting ability of sickled erythrocytes (Chiu *et al.*, 1979, 1981; Lubin *et al.*, 1981). Similar observations have recently been reported for spectrin-free spicules derived from sickle cells and for microvesicles isolated from normal erythrocytes (Westerman *et al.*, 1984). Additionally, there is convincing evidence that transbilayer movement of phosphatidylcholine (PC) is considerably accelerated in sickled erythrocytes compared with unsickled cells (Franck *et al.*, 1983).

Although these observations suggest that phospholipid asymmetry has been altered in those regions of the cell membrane that correspond to the extended spicules on sickled cells and that seem to be free of skeletal proteins (Allan *et al.*, 1982), this has not been shown conclusively. We decided, therefore, to isolate free spicules by reoxygenation of sickled cells as described previously (Allan *et al.*, 1982) and to determine the orientation of their phospholipids using phospholipase A_2 as a probe of phospholipid accessbility.

Abbreviations used: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Hb, haemoglobin; Mops, 4-morpholinepropanesulphonic acid; PLA_2, phospholipase A_2.

Methods

Blood from donors homozygous for HbS was collected in heparinized saline (0.9% NaCl) and erythrocytes were washed four times by centrifugation in 20mM-Mops/NaOH buffer, pH 7.1, containing 0.15M-NaCl (Mops/saline), with removal of buffy coat. Washed erythrocytes were resuspended at ~25% haematocrit in Mops/saline and incubated under N_2 at 37°C in 28g (1oz) McCartney bottles for 90min with regular shaking. At 20min intervals, the samples were flushed with N_2. After 90min, when ~90% sickling was observed, the cells were incubated for a further 5min in the presence of 1mM-Ca^{2+}. Phospholipase A_2 (PLA_2) from bee venom ($10\,\mu g \cdot ml^{-1}$) obtained from

Sigma Chemical Co. (lot nos. 52F0563 and 63F0468) or from Calbiochem (lot no. 102254) was added to the cells with continued incubation at 37°C under N_2. At timed intervals, 0.5ml samples were added to 1.8ml of methanol/chloroform (2:1, v/v) containing 2.5mM-EDTA in order to inactivate the enzyme and extract the lipids. Controls were incubated in the absence of added PLA_2.

Individual phospholipids were separated and quantified as previously described (Allan *et al.*, 1980). Haemolysis was determined by withdrawing 0.2ml samples into 1.8ml of Mops/saline containing 2.5mM-EDTA. After centrifugation at 1000*g* for 5min, the haemoglobin released into the supernatant was quantified by measurement of its absorbance at 410nm.

Free spicules were isolated from sickled erythrocytes as described by Allan *et al.* (1982). Washed spicules were resuspended in Mops/saline containing 1mM-Ca^{2+} and were preincubated at 37°C for 5min. PLA_2 from bee venom ($3\mu g \cdot ml^{-1}$) was added and degradation of phospholipids was measured as described for sickled erythrocytes.

Results

In all experiments, sickled erythrocytes were found to be more susceptible to PLA_2-dependent lysis than were either unsickled cells or normal human erythrocytes when treated with identical concentrations of the enzyme. Thus, whereas release of haemoglobin from sickled cells was always greater than 10% after 2h incubation with the enzyme, release of haemoglobin from unsickled cells was only about 1–2%. These results were unaltered under different conditions of incubation, e.g. using the high-K^+ medium of Lubin *et al.* (1981) or without the use of EDTA to stop PLA_2 activity. As shown in Fig. 1(*a*), after correcting for lysis, >50% of the total PC of sickled cells was broken down by 30min, whereas degradation of PE was <8% and that of PS was zero. This result was also obtained with unsickled cells incubated in air and is little different from results reported previously for normal (HbA) erythrocytes (Zwaal *et al.*, 1975; Haest *et al.*, 1978; Raval & Allan, 1984). [There is good evidence that prior treatment with sphingomyelinase enables more (~20%) of the PE to be revealed on the surface of human erythrocytes by the subsequent action of PLA_2. However, in agreement with other workers (Lubin *et al.*, 1981) we found that consecutive treatment of sickled cells with both enzymes caused unacceptable levels of lysis, so that this dual treatment was not further investigated.] With longer incubation periods, there was a gradual increase in the amounts of PE and PS that were available to the enzyme, but this increase seemed to be largely due to a progressive rise in cell lysis as measured by haemoglobin release. In five similar experiments the relation

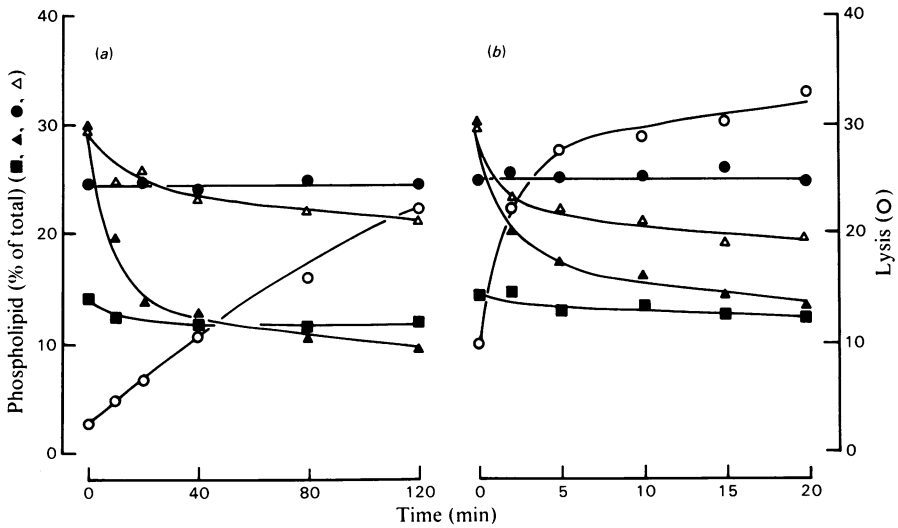

Fig. 1. *Degradation of phospholipids on treatment of (a) sickled erythrocytes and (b) spicules with PLA_2*
Experimental details are given in the Methods section. The amounts of each lipid class (●, sphingomyelin; ▲, ■, PS; and △, PE) were expressed as a percentage of total phospholipid. Lysis (○) was expressed as a percentage of total haemoglobin released in the supernatant after centrifugation at $16000g_{av.}$ for 20min (spicules) or $1000g_{av.}$ for 5min (cells). Results are presented for a single representative experiment in each case, the same batch of cells being used for (*a*) and (*b*).

between percentage breakdown of PE and percentage lysis was linear ($r = 0.97$) and the gradient of the regression line was 1.5 ± 0.5. For PS, the gradient of the regression line was 1.2 ± 0.6 and the regression coefficient was 0.92. Identical results were obtained using the high-K^+ medium employed by Lubin *et al.* (1981).

Experiments involving PLA_2 treatment of free spicules were somewhat disappointing, in that a significant fraction of the spicule population (corresponding to about 25% of the total haemoglobin) seemed to be lysed very rapidly by the enzyme. All attempts to eliminate this lysis, e.g. by altering the composition of the medium, by preparing the spicules as rapidly as possible, or by using different batches of enzyme, failed to suppress this effect of the enzyme. Preliminary evidence suggested that the susceptible fraction of the spicule preparation might correspond to the relatively large spherical elements previously shown to be present (Allan *et al.*, 1982). This would be analogous with our observations (P. J. Raval & D. Allan, unpublished work) that normal erythrocytes that have been made spherical by treatment with Ca^{2+} ionophore A23187 are rapidly lysed when subsequently treated with PLA_2.

Allowing for enzyme-dependent lysis, free spicules gave results rather similar to those given by whole cells (Fig. 1*b*). Samples from six experiments gave a breakdown-versus-lysis regression line with a gradient of 1.2 ± 0.3 ($r = 0.91$) for PS. Curiously, breakdown of PC was decreased in free spicules compared with intact cells, and this result confirms a similar observation made with PLA_2 treatment of microvesicles (Raval & Allan, 1984). It is not known whether this reflects a loss of PC from the outer leaflet in these spectrin-free vesicles or a change in lipid packing that decreases the affinity of the enzyme for PC.

Discussion

The conclusion from these experiments is that breakdown of PE and PS in sickled cells or in free spicules can be explained largely as a consequence of cell lysis and that it is not necessary to invoke explanations depending on a loss of lipid asymmetry. The slight excess of PE and PS breakdown above the lysis values could be due to an entry of PLA_2 (M_r 19000) into partially lysed cells, which is somewhat more rapid than the exit of haemoglobin (M_r 68000) from such cells. There is no indication that absence of spectrin from the free spicules is associated with any enhanced exposure of PE and PS beyond that due to enzyme-dependent lysis. These results are consistent with our recent work, which shows that spectrin-free microvesicles derived from Ca^{2+}/A23187-treated normal cells retain the phospholipid asymmetry of the original cells (Raval & Allan, 1984). However, they do not support the claims of other workers that spectrin is necessary for the maintenance of aminophospholipid asymmetry in erythrocytes (Haest *et al.*, 1978; Haest, 1982; Kumar & Gupta, 1983) or that sickle cells show a loss of aminophospholipid asymmetry when they sickle (Chiu *et al.*, 1979, 1981; Lubin *et al.*, 1981). It is not at present clear why our results differ from those of others using the same enzyme preparation. The crucial difference seems to lie in the amounts of lysis observed; we have repeatedly failed to obtain the very low levels of lysis claimed by Lubin and his collaborators (Lubin *et al.*, 1981) for sickled cells before or after treatment with PLA_2.

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